

Smad 1/5 and Smad 4 Expression Are Necessary for Osteoclast
Differentiation

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Brandon Pei-Han Huang

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Dedication

This thesis is dedicated to my wife and my mother for their love and support.

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Introduction

The human skeleton is structurally unyielding due to its high mineral content, yet highly adaptive as it is dynamically remodeled throughout life to meet our daily needs. The process of bone remodeling is primarily carried out by two types of cells, osteoblasts and osteoclasts. Osteoblasts synthesize the protein matrix that mineralizes to become bone; whereas osteoclasts demineralize and resorb bone. The intricate balance between their activities is critical for bone homeostasis, and any disruption of this harmonious relationship can lead to both systemic and localized diseases, such as osteoporosis, osteolytic malignancies, and periodontitis (Zaidi, 2007). Osteoblasts and osteoclasts are tightly regulated by systemic hormones and locally produced cytokines, such as bone morphogenetic proteins (BMPs) (Biver, Hardouin, & Caverzasio, 2013; Giannoudis, Kanakaris, & Einhorn, 2007). BMPs have long established themselves as direct positive regulators of osteoblastic activity; however, their influences on osteoclasts have been controversial. Recent work from the Gopalakrishnan/Mansky lab has provided convincing evidence that BMP signaling directly upregulates osteoclastogenesis induced by RANKL and M-CSF (Broege et al., 2013; Jensen et al., 2010; Sotillo Rodriguez et al., 2009). Upon binding of the cell surface BMP receptors, BMPs exert their regulatory effects through the activation of two distinct intracellular pathways: the canonical pathway involving Smad proteins and the non-canonical pathway involving the MAP kinases (Biver et al., 2013). The goal of this project is to further investigate the specific role of the canonical Smad pathway of BMP signaling in osteoclastogenesis.

Review of the Literature

The skeleton provides protection for our key organs, at the same time; it serves as a rigid framework to which muscles attach in order to perform our motor activities. While its inner cavity holds a reservoir of bone marrow/stem cells that constantly replenish new blood cells, the mineralized bone itself is the body's major storage of calcium and phosphate for the maintenance of mineral homeostasis in the body (Henriksen, Neutzsky-Wulff, Bonewald, & Karsdal, 2009; Zaidi, 2007). All in all, it is a highly versatile and indispensable organ of our bodies.

Bone Cells

As the weight bearer of the body, the bone is constantly challenged by high load, which inevitably leads to structural damage. Yet, we do not crumble to pieces because of continuous bone remodeling mediated by two key bone cells: osteoblasts and osteoclasts. Bone remodeling describes the process by which old damaged bone is resorbed away by osteoclasts and subsequently replaced with new bone by osteoblasts (Henriksen et al., 2009; Zaidi, 2007). Osteoblasts are of mesenchymal origin and their main function is to produce bone by secreting osteoid (unmineralized protein matrix). As they become embedded in bone, they differentiate into osteocytes. In addition, osteoblasts regulate the bone resorbing cell, osteoclasts, via releasing extracellular mediators, such as RANKL and OPG (Mellis, Itzstein, Helfrich, & Crockett, 2011). Osteoclasts belong to the monocyte/macrophage lineage of hematopoietic origin (Väänänen & Laitala-Leinonen, 2008). Their differentiation begins as mononuclear precursor cells and, through cell-cell

fusion, they become multi-nucleated cells able to resorb bone (Mellis et al., 2011). The balance of osteoblastic and osteoclastic function has a direct effect on the integrity of bone. When osteoclastic activity is too exaggerated, bone pathologies such as osteoporosis, rheumatoid arthritis, periodontitis, and osteolytic malignancies can occur (Yavropoulou & Yovos, 2008).

Osteoclastogenesis

Due to the aforementioned morbidity and mortality associated with overabundance of osteoclastic activity, the process of osteoclast differentiation (osteoclastogenesis) is heavily researched; however, the regulatory mechanisms controlling osteoclastogenesis are still not fully understood at the present time. Starting from the pluripotent hematopoietic stem cells, granulocyte-macrophage colony forming unit (GM-CFU) is the earliest identifiable precursor to osteoclasts (Menaar, Kurihara, & Roodman, 2000). To commit to the osteoclast cell fate, osteoclast progenitor cells depend on the activation of surface receptors, c-FMS and RANK, by M-CSF (macrophage colony stimulating factor) and RANKL (Receptor Activator of Nuclear Factor- κ B ligand), respectively, to promote proliferation and prevent apoptosis. The co-stimulation from M-CSF and RANKL continues to be critical in the differentiation of immature precursor cells to mononuclear TRAP⁺ preosteoclasts as they induce the expression of key transcription factors, such as PU.1, MIF1, c-FOS, and NFATc1. Finally, under the expression of master fusion regulator, DC-STAMP, fusion of mononuclear precursors takes place to form mature multinuclear osteoclasts (reviewed in (Mellis et al., 2011)).

The RANK/RANKL/OPG regulatory axis

Although osteoblasts and osteoclasts have antagonistic effects on bone volume, the traditional model describes osteoblasts as the major cell type to regulate the recruitment and differentiation of osteoclasts through the release of RANKL and OPG. RANKL belongs to the tumor necrosis factor (TNF) superfamily and is normally expressed on the surface of osteoblasts and bone marrow stromal cells to promote osteoclast differentiation upon binding to its receptor RANK on osteoclast precursor cells. However, emerging data recently have supported the idea that the osteocytes, typically described as quiescent cells embedded in bone, actually may be the major source of RANKL to support osteoclastogenesis. Nakashima et al. demonstrated that isolated osteocytes expressed larger quantities of RANKL and supported osteoclast differentiation better than osteoblasts did. They also showed that a lack of RANKL expression specifically from osteocytes led to a severe osteopetrotic phenotype (Nakashima et al., 2011). The loss of RANKL leads to defect in osteoclastogenesis and an osteopetrotic phenotype *in vivo* and *in vitro* (Kong et al., 1999; J. Li et al., 2000). Osteoprotegrin (OPG) act as a decoy receptor that sequesters RANKL from binding RANK when its activation is undesired (Udagawa et al., 2000). Figure 1 shows the model of RANKL/RANK/OPG interaction. As expected, over-expression of OPG leads to a similar phenotype as RANKL deficiency (J. Li et al., 2000). Through modulating the ratio of RANKL and OPG secreted from osteoblasts, systemic hormones, such as parathyroid hormone (PTH), 1,25 (OH)₂ vitamin D₃, calcitonin, and local paracrine and autocrine factors can influence osteoclastogenesis (Boyle, Simonet, & Lacey, 2003).

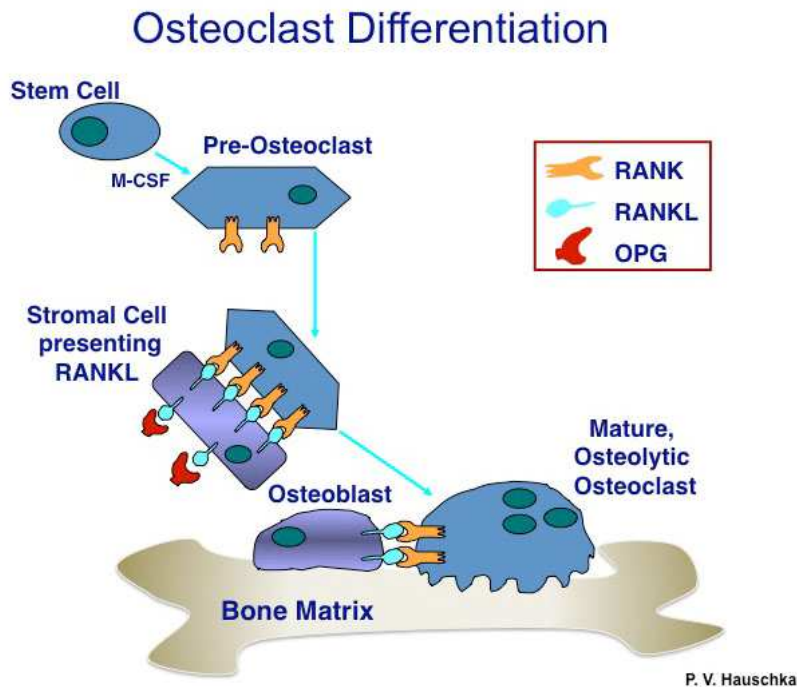


Figure 1. Osteoblast and stromal cells expression RANKL on their cell surface and release OPG into extracellular space. Upon cell-cell contact, RANKL binds RANK on the surface of osteoclast precursors and stimulates osteoclast differentiation and activation. OPG acts as a decoy receptor to prevent binding of RANKL to RANK.

Genes Involved in the Differentiation and Fusion of osteoclast

NFAT (Nuclear Factor of Activated T-cells)

Nuclear Factor of Activated T cells (NFAT) is a family of transcription factors that is determined to be necessary and sufficient for osteoclastogenesis. In vitro studies demonstrated that homozygous knockout of NFATc1 completely disrupts the formation of osteoclast from embryonic stem cells, and ectopic-expression of constitutively active NFATc1 can induce osteoclast differentiation in the absence of normally required

RANKL stimulation (Takayanagi et al., 2002). Upon stimulation by RANKL, increased intracellular Ca^{2+} level activates the Ca^{2+} /calmodulin-dependent calcineurin, which leads to the nuclear translocation of NFATc1 (Takayanagi, 2007). Subsequently, NFATc1 up-regulates the expression of several osteoclast-specific genes, including *cathepsin K* (Ctsk), *acid phosphatase 5* (Acp5), *osteoclast-associated receptor* (OSCAR), and *calcitonin receptor* (CTR) to promote differentiation.

Dendritic Cell-Specific Transmembrane Protein (DCSTAMP) and *the d2 isoform of vacuolar ATPase Vo Domain* (Atp6v0d2)

The cardinal feature of osteoclasts is the multinucleation by cell-cell fusion of mononuclear osteoclasts into a multinuclear osteoclast, which is essential for efficient bone resorption. *Dendritic cell-specific transmembrane protein* (DCSTAMP) is a seven-transmembrane surface protein whereas Atp6v0d2 is a component of the vacuolar ATPase proton pump involved in extracellular acidification (Lee et al., 2006; Miyamoto, 2011). When RANK is activated, NFATc1 positively regulates the expression of fusion genes, *DC-STAMP* and *Atp6v0d2* to promote multinucleation (Kim, Lee, Ha Kim, Choi, & Kim, 2008). *DC-STAMP* deficient mice, both *in vivo* and *in vitro*, have abundant mononuclear osteoclasts with normal expression of osteoclast differentiation markers but are completely void of any multinuclear osteoclasts, indicating that DC-STAMP is specifically required for cell-cell fusion but is not involved in osteoclast differentiation (Yagi et al., 2005). Similarly, *v-ATPase Vo subunit d2*-deficient mice demonstrated an osteopetrotic phenotype due a decreased bone resorptive activity from the lack of

multinuclear osteoclasts (Lee et al., 2006). The fact that bone resorption is not completely lost in DC-STAMP/v-ATPase deficient mice suggests that mononuclear osteoclasts are capable of bone resorption, but at a less efficient level.

Cathepsin K (Ctsk)

Bone resorption is a combination of mineral dissolution by acid and collagen matrix degradation by proteolytic enzymes. Cathepsin K, primarily expressed in osteoclast, is responsible for cleaving type I collagen, the main organic constituent of bone. Pycnodysostosis, a human genetic disorder, exhibits a lack of normal collagen breakdown resulting in sclerotic bone that is susceptible to fracture due to mutations in *Ctsk* and the phenotype can be reproduced in mice deficient in *Ctsk* (Goto, Yamaza, & Tanaka, 2003).

Tartrate-resistant acid phosphatase (TRAP)

Tartrate-resistant acid phosphatase (TRAP), also known as *acid phosphatase 5* (Acp5), is widely recognized as a biochemical marker for osteoclast function and a terminal differentiation marker for mature osteoclasts. It is ubiquitously expressed in many tissues, such as bone, liver, spleen, thymus, and colon. *Acp5*^{-/-} mice demonstrate that the absence of TRAP leads to mild osteopetrosis, associated with reduced osteoclastic activity, which impedes endochondral ossification in the development of long bones causing shortening and deformation of the limb and axial skeleton. In addition, *Acp5*^{-/-} mice also exhibit reduced immune and inflammatory response due to

impaired macrophage function as TRAP is also expressed in macrophages (Hayman & Cox, 2003).

Bone morphogenetic proteins and their signaling pathways

Bone morphogenetic proteins (BMPs) were first discovered by Urist as the osteoinductive component of demineralized bone matrix about 40 years ago. Its essential role in osteoblast differentiation, survival, and endochondral ossification in skeletogenesis, has been firmly established (Biver et al., 2013; X. Li & Cao, 2006). BMPs belong to the transforming growth factor- β (TGF- β) superfamily. Among the fifteen BMPs found in human, BMP 2, 4, and 6 are secreted by osteoblasts and act as autocrine regulators to promote osteogenesis. BMP receptors type I and type II are surface serine/threonine kinases that undergo oligomerization and transactivation upon activation by BMPs.

Smad (signal transducing molecules of TGF β superfamily) is the canonical signaling pathway of BMPs. After forming heteromeric complex with BMP receptor type II, BMP receptor type I initiates Smad signaling via phosphorylation of receptor activated Smads (R-Smads-1, -5, and -8), which when phosphorylated, form a heterodimeric complex with the common Smad (co-Smad 4). The Smad complex subsequently translocates to the nucleus to modulate transcription of BMP target genes (Biver et al., 2013).

Non-canonical pathways under BMPs have also been described, in particular the MAP kinase (MAPK) signaling axis. It has been suggested that different modes of BMP

receptor oligomerization determine the activation of different signaling pathways downstream of BMPs (Nohe et al., 2002). Broege et al. demonstrated that the canonical (Smad) pathway and non-canonical (MAPK) pathways are activated at different time points during osteoclastogenesis. In pre-fusion mononuclear osteoclasts, MAPK pathway is utilized to maintain proliferation, survival, and resorption activity (Fong et al., 2013); whereas, Smad phosphorylation becomes apparent when fusion is about to take place (Broege et al., 2013).

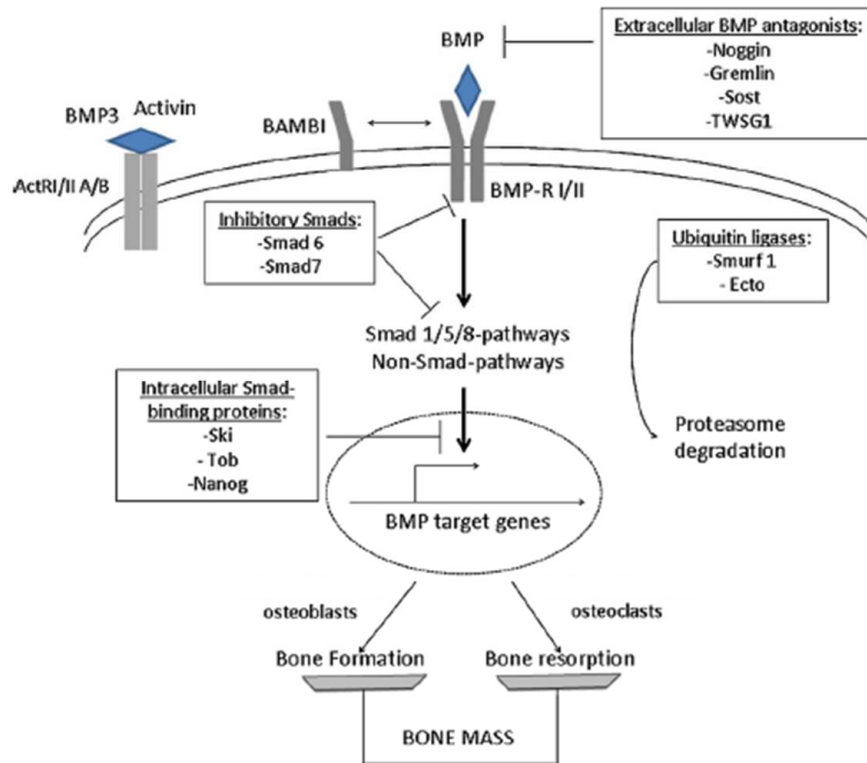


Figure 2. The BMPs and its downstream signaling pathways. BMP signaling can be modulated by various extracellular regulators and intracellular inhibitors. BMPs bind to surface BMP receptor I and II. Through subsequent activation of the canonical Smad and non-canonical Smad-independent pathways, BMPs regulate key genes in osteoblast and osteoclast differentiation.

Bone Morphogenetic Proteins and Osteoclastogenesis

Besides its well established role in promoting osteoblast differentiation and bone formation, BMPs have been shown to promote osteoclastogenesis as well. However, the exact mechanism(s) are still not clear (Giannoudis et al., 2007). BMPs have been demonstrated to indirectly, via an osteoblast-dependent manner, stimulate osteoclastogenesis and bone resorption. Tachi et al demonstrated that BMP-2, only in the presence of $1\alpha,25$ -dihydroxyvitamin D(3), upregulates the expression of RANKL from osteoblasts to promote osteoclastogenesis and fail to do so when co-cultured osteoblasts are not activated by vitamin D (Tachi et al., 2010). Kanatani et al showed that BMP-2 dose dependently stimulated mature osteoclast-like cells only in the presence of stromal cells (Kanatani et al., 1995). Abe et al. demonstrated that osteoblastogenesis is a prerequisite for osteoclastogenesis by employing a BMP inhibitor, noggin, which did not directly act on the osteoclast lineage (Abe et al., 2000).

On the other hand, convincing data has accumulated to challenge the hypothesis that BMPs only affect osteoclast differentiation through an osteoblast-dependent mechanism. Kaneko et al. provided strong evidence that BMPs act directly on the osteoclast lineage. By isolating osteoclasts from long bones of rabbits, they found BMP receptors IA and II on mature osteoclasts. They also demonstrated that BMP-2, without exogenous RANKL, directly stimulated bone resorption and upregulated the expression of *cathepsin K* (Kaneko et al., 2000). Itoh et al. presented consistent findings that BMP receptor type IA is expressed by both precursors and mature osteoclasts, and BMP-2 drastically enhanced the differentiation of isolated osteoclasts induced by RANKL and

M-CSF (Itoh et al., 2001). Data from the Mansky/Gopalakrishnan lab has further confirmed a direct stimulatory mechanism by BMPs. Enhanced BMP signaling due to the loss of *Twisted gastrulation*, an extracellular BMP inhibitor, leads to increased number, size and activity of osteoclasts (Sotillo Rodriguez et al., 2009). Furthermore, Jensen et al. demonstrated that exogenous BMP-2 directly enhanced RANKL-dependent formation of wild type bone marrow derived osteoclasts and confirmed the endogenous expression of, BMP receptors and BMP-2 in osteoclasts (Jensen et al., 2010). More recently, Broege et al. demonstrated that loss of expression of BMP receptor type II in osteoclasts result in an osteopetrotic mouse phenotype primarily due to a change in the noncanonical MAPK signaling by BMP receptors; whereas, the phosphorylation of Smad 1/5 only became detectable in osteoclasts around the time of fusion suggesting that the canonical Smad signaling pathway may be more involved in regulating fusion of the osteoclast precursors (Broege et al., 2013).

Goal of the work

The exact role of Smad signaling in osteoclast differentiation induced by BMP remains to be elucidated, and it is the goal of my thesis project to further characterize the role of Smad 1/5 and Smad 4 in osteoclastogenesis by using knockdown adenoviral CRE recombinase and control osteoclast models.

Hypothesis

The attenuation of the Smad signaling pathway will negatively impact osteoclastogenesis and resorption activity in the presence of RANKL stimulation.

Specific Aims:

1. To determine the effects of Smad 1/5, Smad 4 gene deletion by using a CRE recombinase adenovirus on histomorphometric measurements of osteoclastic differentiation; namely, cell number and cell size (area).
2. To determine the effects of Smad 1/5, Smad 4 gene deletion by using a CRE recombinase adenovirus on osteoclast bone resorption activity.
3. To determine the effects of Smad 1/5, Smad 4 gene deletion by using a CRE recombinase adenovirus on expression of important osteoclastogenesis markers using RT-PCR; namely, *NFATc1*, *DC-STAMP* and *Ctsk*.
4. To determine the necessity of the canonical Smad signaling in BMP enhancement of resorption in mature osteoclast by utilizing dorsomorphin, a specific inhibitor of Smad 1/5/8 phosphorylation.

Materials and Methods

Transgenic mice

Smad4^{fl/fl} and *Smad1/5^{fl/fl}* mice were generated by mating homozygous *Smad4^{fl/fl}* and *Smad1/5^{fl/fl}* mice. *Smad 1/5* floxed mice obtained from Dr. Stephanie Pangas, Baylor College of Medicine, Houston, TX (Pangas et al., 2008) with permission obtained from Dr. Elizabeth Robertson (Oxford University, United Kingdom) and Dr. An Zwijsen (VIB and Center for Human Genetics, KU Leuven, Belgium) who generated the *Smad1^{fl/fl}* and *Smad5^{fl/fl}* mice, respectively in a mixed background of C57Bl/6 and 129SV. *Smad 4* floxed mice were obtained from Dr. Michael O'Connor (University of Minnesota). *Smad 4* floxed mice were originally generated by Dr. Deng (NIH). Mice were in a C57Bl/6 background. The care and use of the animal in this study followed the standards established by the University of Minnesota Institution of Animal Use and Care Committee.

Primary Osteoclast Cultures

Four week old wild type or homozygous floxed mice were sacrificed by CO₂ inhalation for their femurs and tibiae. Bone marrow of the femurs and tibiae was collected and cultured in alpha-MEM media for 24 hours in the presence of 50 ng/ml M-CSF (macrophage-colony stimulating factor) on culture dishes. The non-adherent cells were counted and equal numbers were plated (containing primarily of macrophages and osteoclast precursors) and cultured for 2 additional days in M-CSF. The next day cells were cultured for an additional 5 days in M-CSF and RANKL (30 ng/ml). Osteoclast

resorption was observed on calcium phosphate plates (Corning). The resorption area was observed and captured with light microscopy and the measurements were analyzed using NIH Image J.

Adenoviral infection

Bone marrow macrophages were isolated as described above. Prior stimulation with RANKL, the cells were incubated with 100 MOI of adenovirus (EGFP or CRE expressing) for 3 hours at 37°C in the presence of M-CSF. After 3 hours, media containing adenovirus was removed and cells were fed with M-CSF and RANKL (30 ng/ml). Cells were cultured until multinuclear cells appeared in the control infected wells. RNA was extracted for use in qRT-PCR, protein was extracted for western blotting, or cells were stained for TRAP.

TRAP Staining and Histomorphometric Analysis

Post-infection primary osteoclast cultures were stained for TRAP activity by fixing in 4% paraformaldehyde, washing by PBS and staining with acid phosphoric reagents with tartrate (5 mg Naphthol AS-Mx phosphate, 0.5 l N,N-dimethyl formamide, 50 ml acetic acid buffer (1 ml acetic acid, 6.8 g sodium acetate trihydrate 11.5 g sodium tartarate in 1 L water), and 25 mg Fast Violet LB salt]. Cells were photographed at 4X and 10X magnifications. All TRAP-positive osteoclasts with three or more nuclei were included and measured by using NIH Image J software. The average area and number of a given 10X magnified field was recorded.

Quantitative real-time PCR

To assess the level of differentiation and function of osteoclasts, RT-PCR was employed to quantify the expression of the following genetic markers (*GAPDH*, *L4*, *DCSTAMP*, *NFATc1*, *Smad1*, *Smad4*, *Smad5* and *cathepsin K*). Total RNA was extracted from osteoclasts using TRIzol reagent (Invitrogen Life Technologies) and quantitated by UV spectroscopy. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-rad) with 1 ug of extracted RNA. The real time-PCR reaction was performed mixing 1 ul of the cDNA and 2X SYBR green (Bio-rad IQ SYBRGreen Supermix) using MyiQ Single-Color real-time PCR Detection System (Bio-rad) in a MX 3000P QPCR System (Stratagene). Amplified genetic markers were normalized to housekeeping genes, *GAPDH* or *L4*. Primer sequences for the genetic markers are listed as follows:

GAPDH Forward 5' TGC ACC ACC AAC TGC TTA 3' Reverse 5' GAT GCA GGG ATG ATG TTC 3', *L4* Forward 5' CCT TCT CTG GAA CAA CCT TCT CG 3' Reverse 5' AAG ATG ATG AAC ACC GAC CTT AGC 3', *DC-STAMP* Forward 5' CAG ACT CCC AAA TGC TGG AT 3' Reverse 5' CTT GTG GAG GAA CCT AAG CG 3', *NFATc1* Forward 5' TCA TCC TGT CCA ACA CCA AA 3' Reverse 5' TCA CCC TGG TGT TCT TCC TC 3', *Smad1* Forward 5' ATG AAT GTG ACC AGC TTG TTT 3' Reverse 5' CTG CTT GGA ACC AAA TGG GAA 3', *Smad5* Forward 5' GGA ACC TGA GCC ACA ATG AA 3' Reverse 5' CTT GCT GGG GAG TTG GGA TA 3', *Smad4* Forward 5' GTG ACG TTT GGG TCA GGT GC 3' Reverse 5' TAT GAA CAG CGT CGC CAG GT 3', *Cathepsin K* Forward 5' AGG GAA GCA AGC ACT GGA TA 3', Reverse 5' GCT GGC TGG AAT CAC ATC TT 3'.

Western Blot

To determine the level of total Smad 1/5 in osteoclasts, total cell lysate were obtained by lysing osteoclasts in modified RIPA buffer in the presence of phosphatase and protease (Pierce) inhibitors. Total protein extract was obtained by centrifuging crude cell lysate at 12,000 rpm at 4 °C, resuspended in SDS-PAGE sample buffer, and resolved by SDS-PAGE. Resolved proteins were transferred to PVDF membrane (Millipore) and immunoblotted against total Smad1/5/8 antibody (Santa Cruz) or total Smad 4 (Cell Signaling) and subsequently horseradish peroxidase-conjugated secondary antibodies. Immunoreactive protein bands were detected using ECL Plus system (GE Health Systems).

Resorption Assays

Primary osteoclasts were plated on Corning Osteo Assay Surface plates at a density of 100,000 cells per well. Cells were allowed to fully differentiate. For resorption assays with BMP2 or dorsomorphin, osteoclasts were allowed to differentiate till day 4. Differentiation medium was supplemented with 50 ng/ml BMP2 (R and D Systems) or 1200 nM dorsomorphin (Sigma) overnight. For all the resorption assays, the media was completely removed on day 5 and 100µL/well of 10% bleach was added and allowed to incubate at room temperature for 5 minutes. The bleach solution was then aspirated and the wells were washed twice with 150µL of dH₂O. The plate was then allowed to air dry completely at room temperature for 3-5 hours. The wells were observed at 10x magnification for the

formation of resorption pits and images were captured with light microscopy.

Images were measured and analyzed using NIH Image J.

Statistical Analysis

All experiments were repeated three times independently. Real-time PCR were performed in duplicates or triplicates and the results were expressed as mean \pm standard deviation. All statistical analyses were done by Prism 5 (Graphpad Software). Student's *t*-test was used to test the significance between different test groups. $P < 0.05$ indicates significance.

Results

In order to determine the significance of the canonical Smad signaling pathway for osteoclastogenesis, transgenic mice that have *Smad1/5 flox/flox* and *Smad4 flox/flox* genes were generated to allow selective deletion of these genes and their protein products. Bone marrow monocytes (BMMs; osteoclast precursors) were isolated from transgenic mice as our study model. The rationale for knocking down only Smad 1/5 instead of all three R-Smads (1/5/8) is that osteoclasts predominantly express Smad 1 and 5 as shown in Figure 3. BMMs were infected with either a control adenovirus expressing EGFP or an adenovirus expression CRE recombinase. It was confirmed by real time RT-PCR and Western blot that *Smad 1/5* (Figure 4) and *Smad 4* (Figure 5) expression was successfully knocked down in osteoclasts infected with the CRE recombinase adenovirus as compared to the control group.

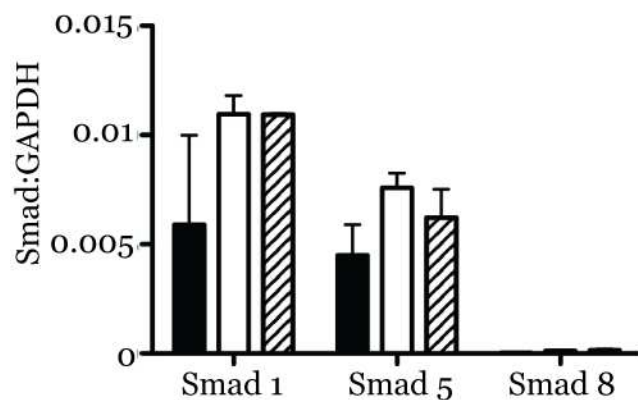


Figure 3. *Smad 1 and 5 are the predominant R-Smads expressed in osteoclasts.* RNA was extracted from wild type BMMs and Smad1/5/8 expression was quantified by RT-PCR. Error Bar represents standard deviation.

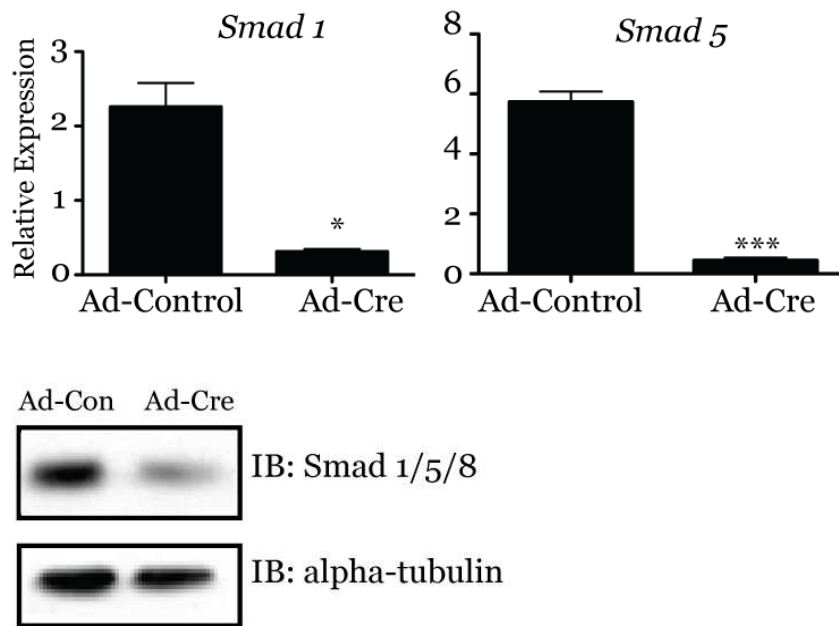


Figure 4. Smad 1/5 mRNA and protein expression was successfully knocked down by CRE recombinase expressing adenovirus as demonstrated by RT-PCR (top) and Western blot (bottom). Ad-control (control expressing EGFP). Ad-CRE (CRE recombinase expressing adenovirus). Error bars represent standard deviation. *=p<0.05. ***=p<0.001.

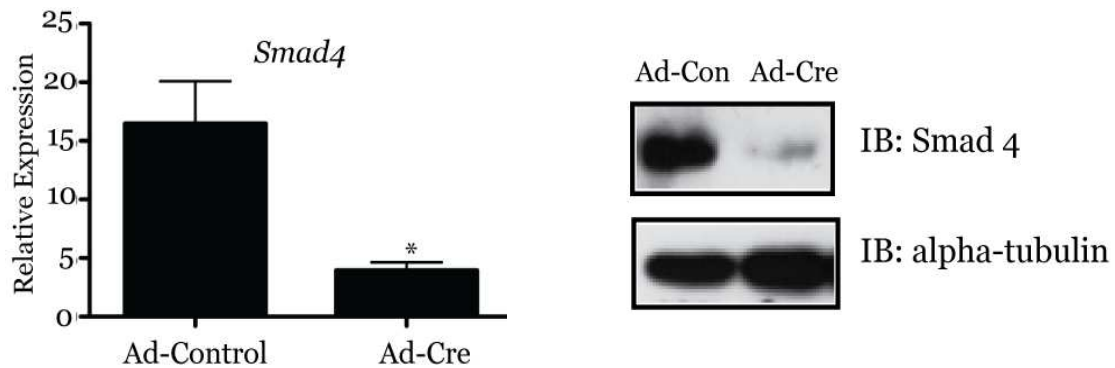


Figure 5. Smad 4 mRNA and protein expression was successfully knocked down by CRE expressing adenovirus as demonstrated by RT-PCR (left) and Western blot (right). Error bars represent standard deviation. *=p<0.05.

Specific Aim 1:

To determine the impact of Smad 1/5 and Smad 4 gene deletion by using a CRE recombinase adenovirus on histomorphometric measurements of osteoclastic differentiation; namely, cell number and cell size (area).

The first aim was to determine the functional impact of Smad 1/5 and Smad 4 knockdown on osteoclastogenesis. BMMs infected with either control or CRE expressing adenoviruses were differentiated in the presence of M-CSF and RANKL for 5 days. Osteoclast cultures were fixed and stained for TRAP for histomorphometric measurements, namely cell number and cell size. As compared to control group, osteoclasts culture deficient in Smad 1/5 expression (Ad-CRE) had less numerous and smaller multinucleated osteoclasts (Figure 6). Likewise, when Smad 4 expression was knocked down in BMMs, TRAP positive multinucleated osteoclasts were less numerous (half as many when compared to control) and smaller (two and half fold smaller when compared to control) (Figure 7).

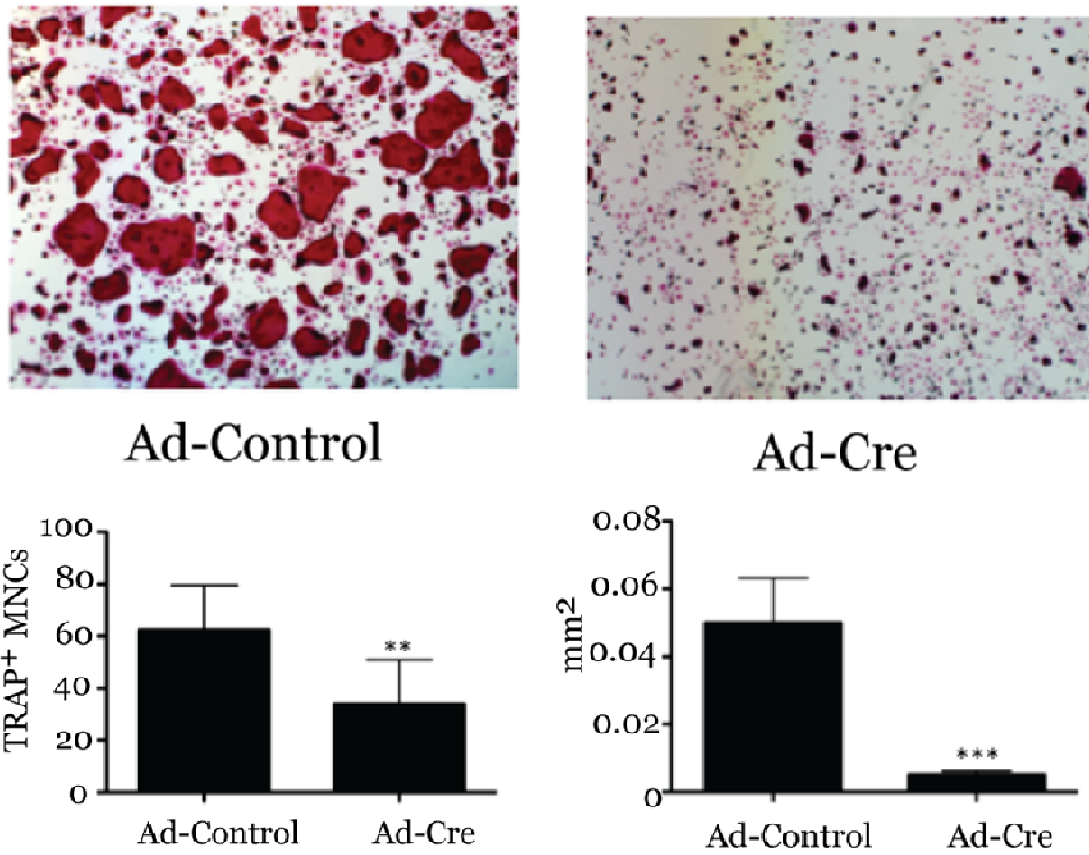


Figure 6. Smad1/5 expression is required for osteoclast differentiation. BMMs were cultured from *Smad1/5*^{fl/fl} mice and infected with either a control (Ad-Control) or CRE expression adenovirus (Ad-CRE). They were stimulated by M-CSF and RANKL, TRAP stained and imaged (top left and top right). TRAP-positive cells were characterized by number (bottom left) and size (bottom right). Values represent the mean with error bar representing standard deviation. *=p<0.05, **=p<0.005, ***=p<0.001.

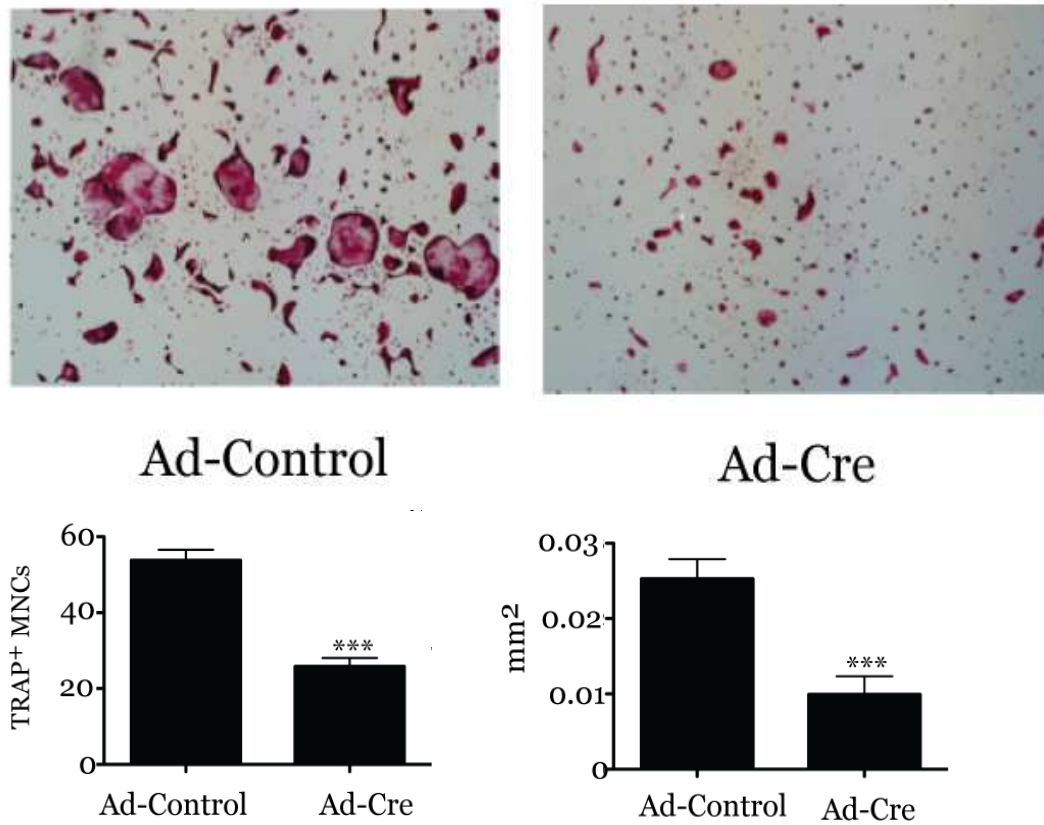


Figure 7. Smad4 expression is required for osteoclast differentiation. BMMs were cultured from *Smad4^{fl/fl}* mice and infected with either a control (Ad-Control) or CRE expression adenovirus (Ad-CRE). They were stimulated by M-CSF and RANKL, TRAP stained and imaged (top left and top right). TRAP-positive cells were characterized by number (bottom left) and size (bottom right). Values represent the mean with error bar representing standard deviation. ***= $p < 0.001$.

Specific Aim 2:

To determine the effects of Smad 1/5, Smad 4 gene deletion by using a CRE recombinase adenovirus on osteoclast bone resorption activity.

Kaneko et al. demonstrated that BMP2 and BMP4 stimulation increased bone resorption activity of rabbit osteoclasts (Kaneko et al., 2000), however, the study did not specify if the increase in osteoclastic activity was due to the canonical or non-canonical pathway under BMP signaling. Broege et al. and Fong et al. demonstrated that when BMPs signal through the non-canonical MAPK pathways, bone resorption is stimulated in mature osteoclasts (Broege et al., 2013; Fong et al., 2013). To determine the effects of deleting Smad expression on osteoclast activity, *Smad 1/5^{fl/fl}* and *Smad 4^{fl/fl}* BMMs were infected by control and CRE adenovirus and cultured on calcium phosphate coated plates in the presence of M-CSF and RANKL for 5 days. Calcium phosphate plates were imaged and percent area resorbed was quantified. Smad 1/5 deficiency in osteoclasts led to a decrease in the number of resorption pits and lower percent area resorbed (Figure 8). Similarly, Smad 4 deficient osteoclasts demonstrated a reduced level of resorption activity as demonstrated by resorption pit and percent resorbed area (Figure 9). Smad 4 Ad-CRE group had a 28-fold decrease in the percent area resorbed.

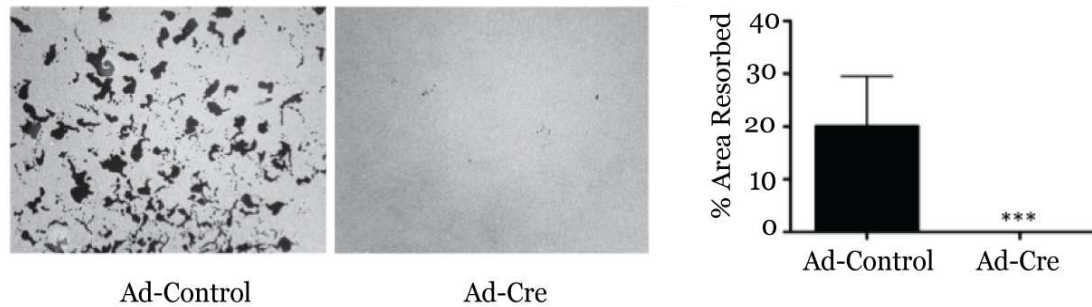


Figure 8. Osteoclast resorption activity is reduced when Smad 1/5 is knocked down. BMMs from *Smad 1/5^{fl/fl}* mice were infected with control (Ad-Control) or CRE expressing (Ad-CRE) adenovirus and cultured on calcium phosphate coated plates with M-CSF and RANKL. Representative images of calcium phosphate coated plates (left) and percent area resorbed quantified (right). Values represent the mean. Error bars represent standard deviation. ***=p<0.001.

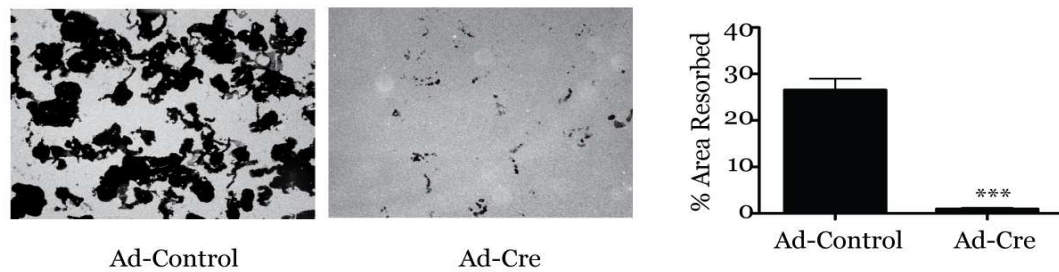


Figure 9. Osteoclast resorption activity is reduced when Smad 4 is knocked down. BMMs from *Smad 4^{fl/fl}* mice were infected with control (Ad-Control) or CRE expressing (Ad-CRE) adenovirus and cultured on calcium phosphate coated plates with M-CSF and RANKL. Representative images of calcium phosphate coated plates (left) and percent area resorbed quantified (right). Values represent the mean. Error bars represent standard deviation. ***=p<0.001

Specific Aim 3:

To determine the effects of Smad 1/5, Smad 4 gene attenuation by using a CRE recombinase adenovirus on expression of important osteoclastogenesis markers using RT-PCR; namely, *Nfatc1*, *DCSTAMP* and *Cathepsin K*.

To investigate the possible causes of reduced osteoclast resorption activity, the changes in the expression of key osteoclast markers, such as *Nfatc1*, *DCSTAMP*, and *Cathepsin K*, was measured in osteoclasts with attenuated of Smad 1/5 and Smad 4 expression. Osteoclasts were infected with control or CRE expression adenovirus under the stimulation of M-CSF and RANKL. RNA was extracted from osteoclast lysate and genes of interest were quantified by RT-PCR against a house keeping gene, *GAPDH*, whose expression remains constant under our experimental treatment. In *Smad 1/5^{fl/fl}* osteoclasts, the expression of *Nfatc1* was not significantly different between control and CRE. However, significant reduction of *DCSTAMP* and *Cathepsin K* expression was observed when Smad 1/5 expression was attenuated. In the Smad1/5 deficient osteoclasts, *DCSTAMP* and *Cathepsin K* expression is reduced 2-fold and 2.5-fold, respectively (Figure 10). In the *Smad 4^{fl/fl}* osteoclasts, a significant decrease in the expression of *Nfatc1*, *DCSTAMP* and *Cathepsin K*, was observed compared to the control group. *Nfatc1* had a 12-fold decrease, *DCSTAMP* had a 3-fold decrease, and *Cathepsin K* had a 4-fold decrease compared to the control cells (Figure 11).

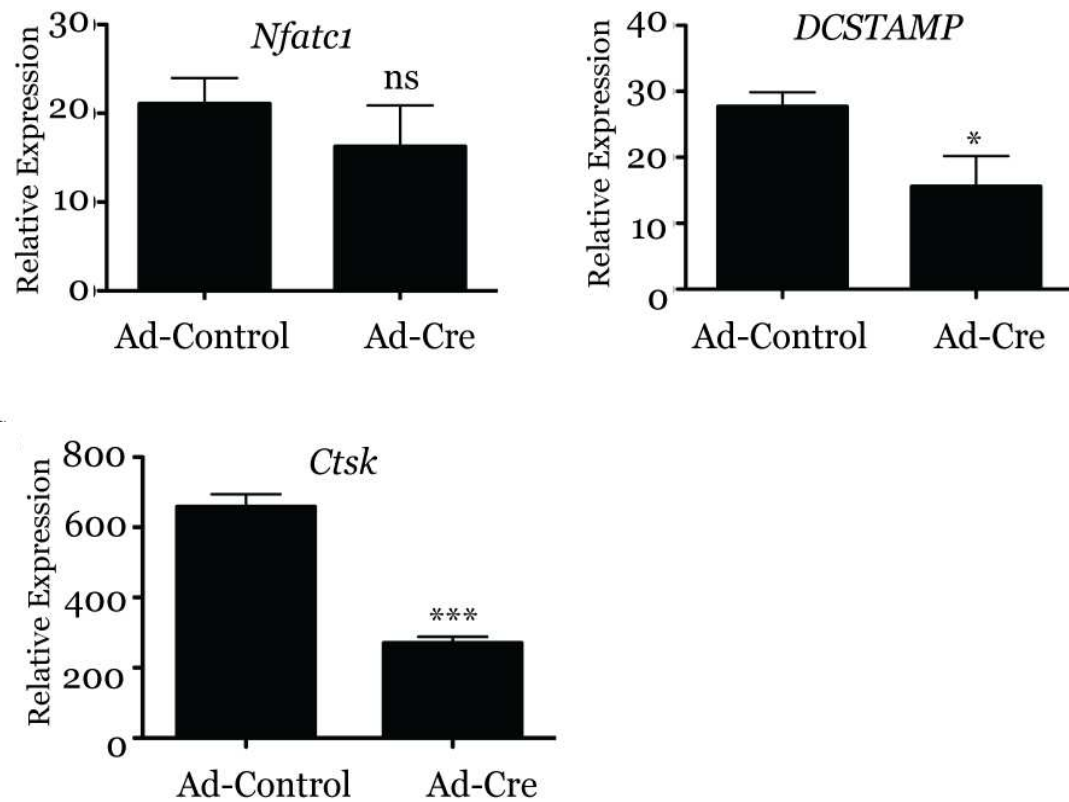


Figure 10. The expression of osteoclast differentiation markers were reduced in Smad 1/5 deficient osteoclasts. RNA was extracted from osteoclasts infected by control (Ad-Control) or CRE (Ad-CRE) expressing adenovirus and *Nfatc1*, *DCSTAMP*, and *Cathepsin K* expression levels were measured by RT-PCR. Values represent the mean. Error bars represent the standard deviation. ns= not significant. *=p<0.05. ***=p<0.001.

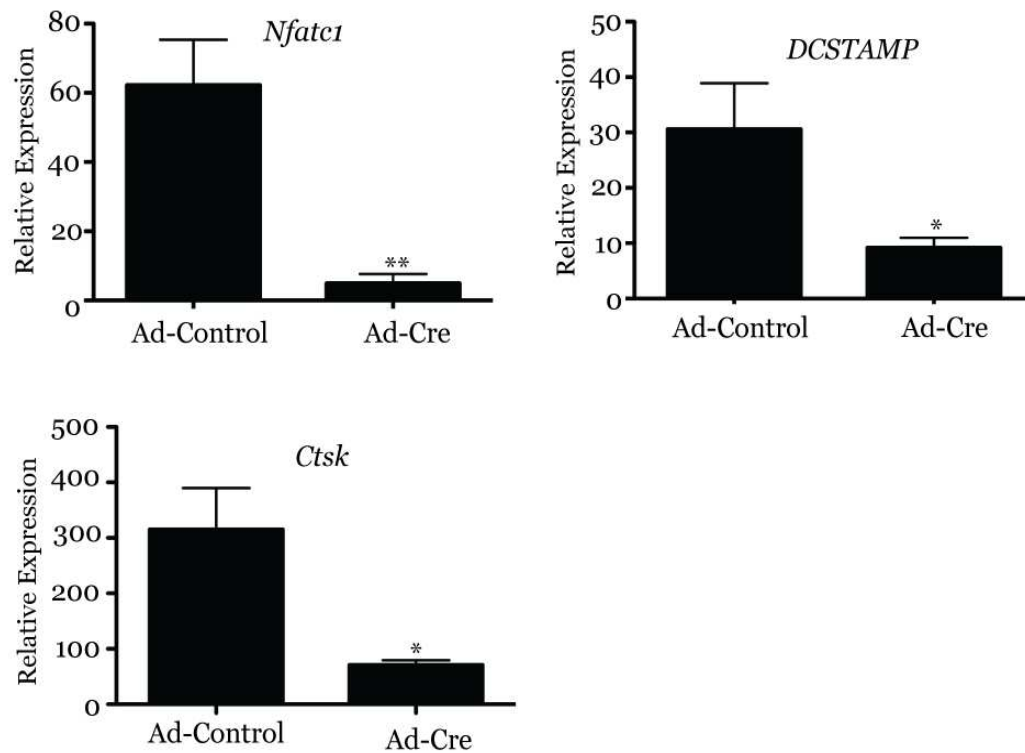


Figure 11. The expression of osteoclast differentiation markers were reduced in Smad 4 deficient osteoclasts. RNA was extracted from osteoclasts infected by control (Ad-Control) or CRE (Ad-CRE) expressing adenovirus and *Nfatc1*, *DCSTAMP*, and *Cathepsin K* expression levels were measured by RT-PCR. Values represent the mean. Error bars represent the standard deviation. *=p<0.05. **=p<0.005.

Specific Aim 4:

To determine the necessity of the canonical Smad signaling in BMP enhancement of resorption in mature osteoclast by utilizing dorsomorphin, a specific inhibitor of Smad 1/5/8 phosphorylation.

The above data demonstrated that osteoclasts in Smad 4 and Smad 1/5 deficient mice were smaller and mostly mononucleated and exhibited a reduced ability to resorb calcium phosphate plates. This is a consistent finding with the literature where Li et al. demonstrated that mononuclear osteoclasts are capable of resorption, but at a lower efficiency than multinuclear osteoclasts (Lee et al., 2006). In attempt to explain if the reduction of calcium phosphate plate resorption in Smad signaling deficient mice is caused by a decrease in osteoclast differentiation or osteoclast activity, normal osteoclasts were differentiated for 5 days into fully mature osteoclasts and then treated with RANKL or RANKL with exogenous BMP2 for 24 hours. Resorptive activity of mature osteoclasts was augmented by BMP2 stimulation as shown by an increase in the total area and total number of resorption pits (Figure 12, top).

To further explore if the canonical Smad signaling pathway mediates the BMP2-induced enhancement of resorption, mature osteoclasts were treated with RANKL with DMSO (control vehicle) or RANKL with dorsomorphin, a known Smad1/5/8 phosphorylation inhibitor. It was found that dorsomorphin treatment significantly reduced the total area, total number and average size of resorption pits (Figure 12, bottom).

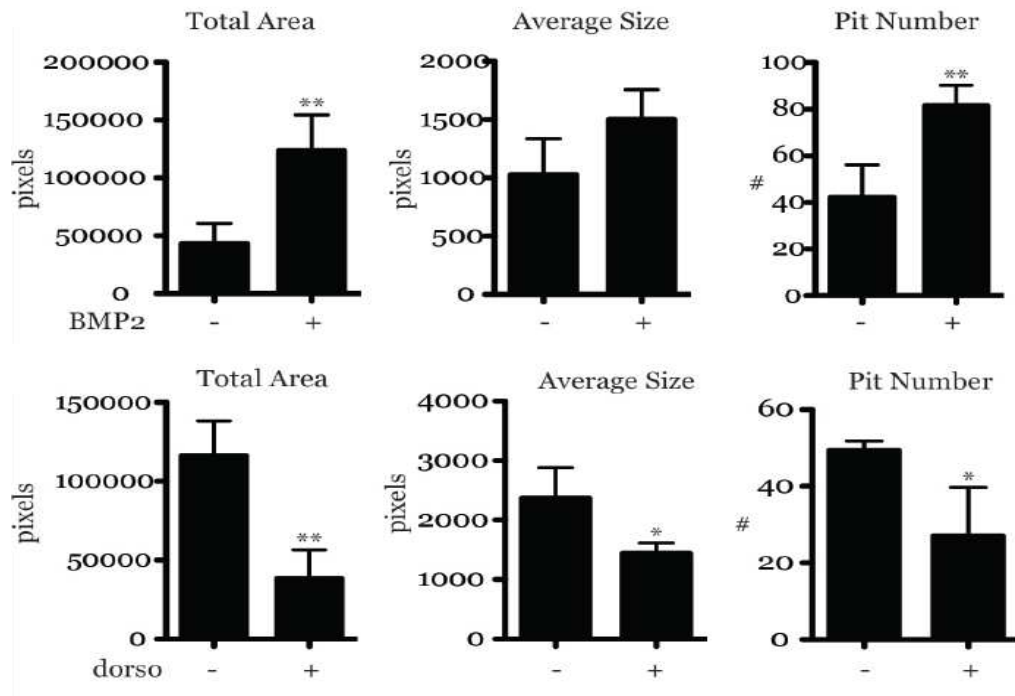


Figure 12. Activity of mature osteoclasts is regulated by canonical Smad signaling when stimulated by BMP2. BMMs from wild type mice were cultured and plated on calcium phosphate coated plates in the presence of M-CSF and RANKL until osteoclasts fuse to become multinucleated cells. (Top panels) Multinuclear osteoclasts were then stimulated in media containing either M-CSF and RANKL or M-CSF and RANKL and BMP2 (50 ng/ml) for 24 hours. (Bottom Panels) Multinucleated osteoclasts were treated for 24 hours with either M-CSF + RANKL +DMSO or M-CSF + RANKL + Dorsomorphin (1200nM). Total area resorbed, average size of pits and number of pits were quantitated. Total resorption area, average size and number of resorption pits were quantified. Values represent the mean. Error bars represent standard deviation. *=p<0.05. **=p<0.001.

Discussion

My research project focused on exploring the role of the BMP canonical signaling pathway in osteoclastogenesis and resorption activity. Specifically, the expression of Smad 4 and Smad 1/5 were selectively attenuated utilizing transgenic mice and recombinase expressing adenovirus. We successfully showed that the expression of Smad 4 and Smad 1/5 are necessary for osteoclast differentiation. Bone marrow monocytes from both Smad 4 and Smad 1/5 deficiency mice differentiated less efficiently as shown by a decrease in TRAP+ mononuclear osteoclasts that fused poorly as demonstrated by smaller multinuclear osteoclasts, when compared to control group. When osteoclasts are stimulated by BMP, Smad 1/5 and Smad 4 form a transcription complex that relocates to the nucleus to regulate gene expression involved in osteoclastogenesis and activation. When Smad 4 and Smad1/5 expression was attenuated, changes in the expression of key osteoclast differentiation markers were observed. *DCSTAMP* and *Cathepsin K* expression were decreased in both Smad 1/5 and Smad 4 deficient osteoclasts; whereas, *Nfatc1* expression was only reduced in Smad 4 knockdown osteoclasts, but not in Smad 1/5 knockdown osteoclasts. Furthermore, consistent with the morphologic and genetic changes, Smad 4 and Smad 1/5 deficient osteoclasts demonstrated less resorption activity on calcium phosphate plates. Finally, BMP2 and dorsomorphin, through their respective positive and negative effects on Smad proteins, are capable of modulating the resorption activity of mature osteoclasts. These data strongly support our hypothesis that “The attenuation of the Smad signaling pathway will negatively impact osteoclastogenesis and resorption activity in the presence of RANKL stimulation”.

Previous data from Rodriguez et al. demonstrated that increasing BMP signaling by attenuation of the expression of a BMP inhibitor, *Twisted gastrulation*, leads to an increase in number and size of wild type osteoclasts (Sotillo Rodriguez et al., 2009). It was noted that Smad 1/5/8 were phosphorylated to a higher level in those large multinucleated osteoclasts when compared to wild type mice. The increase in phosphorylation of Smad 1/5/8 appears to coincide with the time when mononuclear osteoclasts begin to fuse. In addition, noggin, a BMP antagonist, only successfully inhibited osteoclast differentiation prior to the time of fusion (Jensen et al., 2010). In combination of our finding that a reduction in the expression of Smad 1/5 leads to smaller and fewer of multinucleated osteoclasts, it appears that Smad 1/5 is necessary for the fusion of mononuclear osteoclasts. Broege et al. successfully demonstrated that the MAPK non-canonical pathway under BMP signaling is necessary for osteoclast differentiation (Broege et al., 2013). Our data in this study further confirms the importance of BMP signaling in osteoclastogenesis that the Smad canonical pathway also is necessary for osteoclast differentiation.

Smad 4 and Smad 1/5 null osteoclasts exhibited similar phenotypes for most of the parameters measured in this project. However, their influence on the expression of the *Nfatc1* was different in that only Smad 4 deficient osteoclasts showed a reduction in *Nfatc1* expression. Smad 4 is named the common-Smad because it is shared by both BMP and TGF- β signaling. Abundant evidence from the literature supports the regulatory role of TGF- β in osteoblasts and osteoclasts. TGF- β has been demonstrated to be required for monocyte to commit to the osteoclast lineage (Karsdal et al., 2003) and it also

enhances osteoclast formation induced by RANKL (Fuller, Lean, Bayley, Wani, & Chambers, 2000). The dual role of Smad 4 in BMP and TGF- β may provide an explanation for the additional reduction of *Nfatc1* expression observed in Smad 4 deficient osteoclasts when compared to Smad 1/5 deficient osteoclasts. It can be speculated that TGF- β signaling through Smad 4 exerts more regulation on *Nfatc1* expression whereas BMP signaling through Smad 1/5 regulates genes involved in fusion, such as *DC-STAMP*. In fact, osteoclasts treated with dorsomorphin (inhibitor of Smad1/5/8 phosphorylation) showed a reduction in *DC-STAMP* expression (Broege et al., 2013). Osteoclasts had an increase in *Nfatc1* expression when treated with M-CSF, RANKL and TGF- β instead of M-CSF and RANKL alone (Fox, Evans, & Lovibond, 2008). Therefore, a change in TGF- β signaling in Smad 4 deficient osteoclasts may have led to the reduction of *Nfatc1* expression observed in our data; whereas, Smad 1/5 deficient osteoclasts have no effect on the expression of *Nfatc1*.

BMP9 has been shown to enhance the ability of mature osteoclast to resorb bone. It was demonstrated that this effect of BMP9 is mediated through the non-canonical BMP signaling, in particular the ERK signaling pathway was activated by BMP9 (Fong et al., 2013). In our study, we demonstrated that BMP2 also augments calcium phosphate resorption when added to mature osteoclast cultures and BMP2's effect is mediated through the canonical Smad signaling. This finding provides evidence that Smad signaling not only has a role in regulating mononuclear osteoclast fusion, but also controls osteoclast resorption activity.

Conclusion

Diseases involving uncontrolled bone resorption cause significant socio-economic burden and suffering. It is, therefore, a pressing matter to understand how osteoclast differentiation and activity are regulated to devise appropriate therapy to tackle the diseases. In addition, there has been a steady paradigm shift in the interest of orthodontic research from appliance mechanics to biology-based therapies, such as accelerated osteogenic orthodontics (commonly known as Wilckodontics) and AcceleDent®. Locally delivered gene-therapy (local RANKL gene transfer) and pharmacological approaches (local injection of bisphosphonates) have specifically targeted regional osteoclast differentiation and activity in the alveolar bone in attempt to modulate orthodontic tooth movement (Kanzaki et al., 2006; Rinchuse, Sosovicka, Robison, & Pendleton, 2007). It is obvious that more knowledge of how osteoclast differentiation and activity are regulated is necessary for the advancement of biology-based orthodontic therapies. BMP signaling provides a new avenue for the regulation of osteoclast differentiation and may be a potential therapeutic target. From this study, we have demonstrated that Smad 1/5 and Smad 4 of the canonical BMP signaling are essential for osteoclast differentiation and activity and they are the mediators for the enhancement of osteoclast resorption when stimulated by BMP2.

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